Supporting Information

Reduction in (pro-)inflammatory responses of lung cells exposed *in vitro* to diesel exhaust treated with a non-catalyzed diesel particle filter

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Contents

Preparation of the triple cell co-cultures

Triple cell co-cultures were prepared according an improved version of the protocol described by Rothen-Rutishauser et al. (2005) and Blank et al. (2007)

16HBE14o$^-$ cell line

Human bronchial epithelial cells (cell line 16HBE14o$^-$, kindly provided by Dr. Gruenert, University of California, San Francisco) were cultured in minimum essential medium (MEM, with Earle's Salts, without L-Glutamine; Gibco) supplemented with 10% fetal calf serum (PAA Laboratories, Lucerna-Chem), 2 mM L-Glutamine (LabForce), 100 units/ml penicillin G (Gibco) and 100 µg/ml streptomycin sulfate (Gibco) at 37°C in a 5% CO_2 humidified atmosphere. Tissue culture flasks were treated with fibronectin coating solution containing 0.1 mg/ml albumin from bovine serum (Sigma-Aldrich), 0.03 mg/ml bovine collagen Type I (BD Biosciences) and 0.01 mg/ml human fibronectin (BD Biosciences) in basal medium eagle (Sigma-Aldrich). Six days before co-culture composition, the cells were seeded with a density of 10^6 cells per 6 well plate insert (surface area of 4.2 cm^2, pores with 3.0 µm in diameter, high pore density PET membranes for 6-well plates; BD Falcon) and grown with 3 and 2 ml culture medium in the lower and upper compartment respectively. A medium change was performed after three days.

Human blood derived macrophages and dendritic cells

To obtain monocyte-derived macrophages (MDMs) and monocyte-derived dendritic cells (MDDCs), peripheral blood monocytes were isolated from buffy coats (blood donation service Bern) using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's manual. After isolation, the monocytes were kept in Rosswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% fetal calf serum (PAA Laboratories), 2 mM L-Glutamine (LabForce), 100 units/ml penicillin G (Gibco) and 100 µg/ml streptomycin sulfate (Gibco) at 37°C in a 5% CO_2 humidified atmosphere. For the differentiation to dendritic cells, 10 ng GM-CSF (R&D systems) and 10 ng interleukin (IL)-4 (Sigma-Aldrich) were added per ml. For the differentiation to macrophages, no growth factors were added. The cells were allowed to differentiate for 5 days before they were used for co-culture composition

Co-culture composition and preparation for exposure

For co-culture composition, the inserts containing 16HBE14o- cells were placed upside-down in a petri-dish and $25x10^4$ MDDCs in 300 µl medium were given on the membrane. The cells were allowed to adhere during 1 hour at 37°C in a 5% CO_2 humidified atmosphere and the inserts were placed into 6 well plates containing 3 ml supplemented RPMI 1640. 2 ml supplemented RPMI 1640 containing $5x10^4$ MDMs were given into the inserts and the cell cultures were kept at 37°C in a 5% CO_2 humidified atmosphere for 24 hours. Per 100 epithelial cells originally seeded onto the insert membranes, 5 macrophages and 25 dendritic cells were added. The number of epithelial cells roughly tripled between seeding and co-culture composition. For dendritic cells it was found that more than 90% attached to the insert membrane (data not shown), for

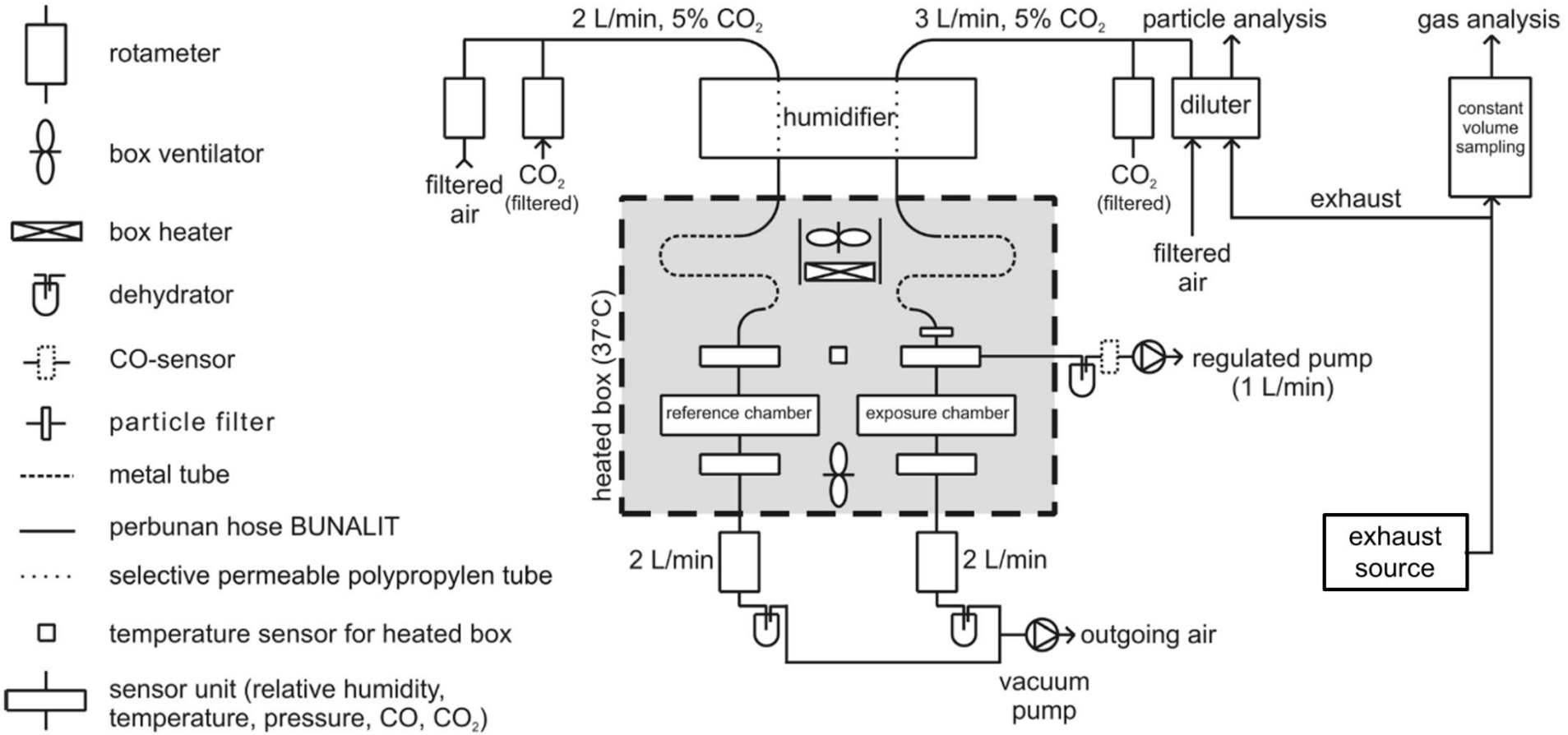
macrophages this was not tested. 24 hours before exposure, the cell cultures were placed into 6 well plates containing 1.2 ml supplemented RPMI 1640 and kept at 37°C in a 5% CO_2 humidified atmosphere. No medium was added to the upper compartment.

Quantification of total reduced GSH

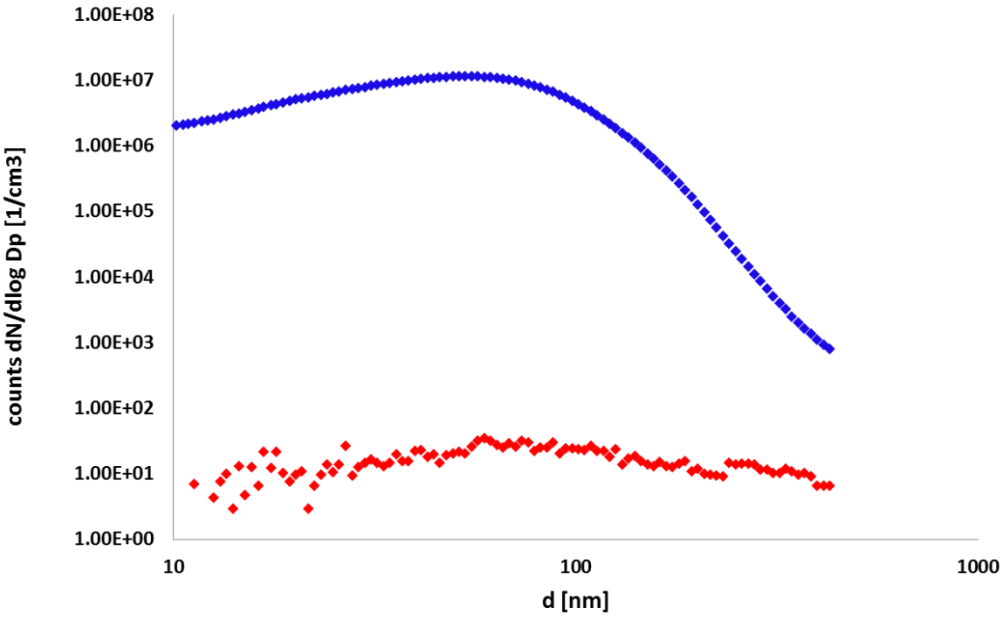
A volume of fixing solution (50 mM 2-(*N*-morpholino)ethanesulfonic acid **(**MES); Sigma-Aldrich, 1 mM ethylenediaminetetraacetic acid **(**EDTA); Sigma-Aldrich) equal to the culture medium volume was added to the complete cell cultures and the cells were lysed by scraping them from the membrane. Insoluble material was removed by centrifugation for 15 min at 10,000 x *g* and 4°C. The soluble fraction of the cell lysate was deproteinated by addition of one volume of a 1.25 M solution of meta-phosphoric acid in water (sigma Aldrich), incubation at room temperature for 5 minutes and centrifugation at 10,000 x *g* at 4°C for five minutes. The samples were then stored at -80°c until further use. Deproteination was completed by addition of 50 µL/mL 4 M triethanolamine. Quantification of total GSH in the obtained solution was performed according to the manufacturers' guidelines (end-point measurement).

Real-time RT-PCR

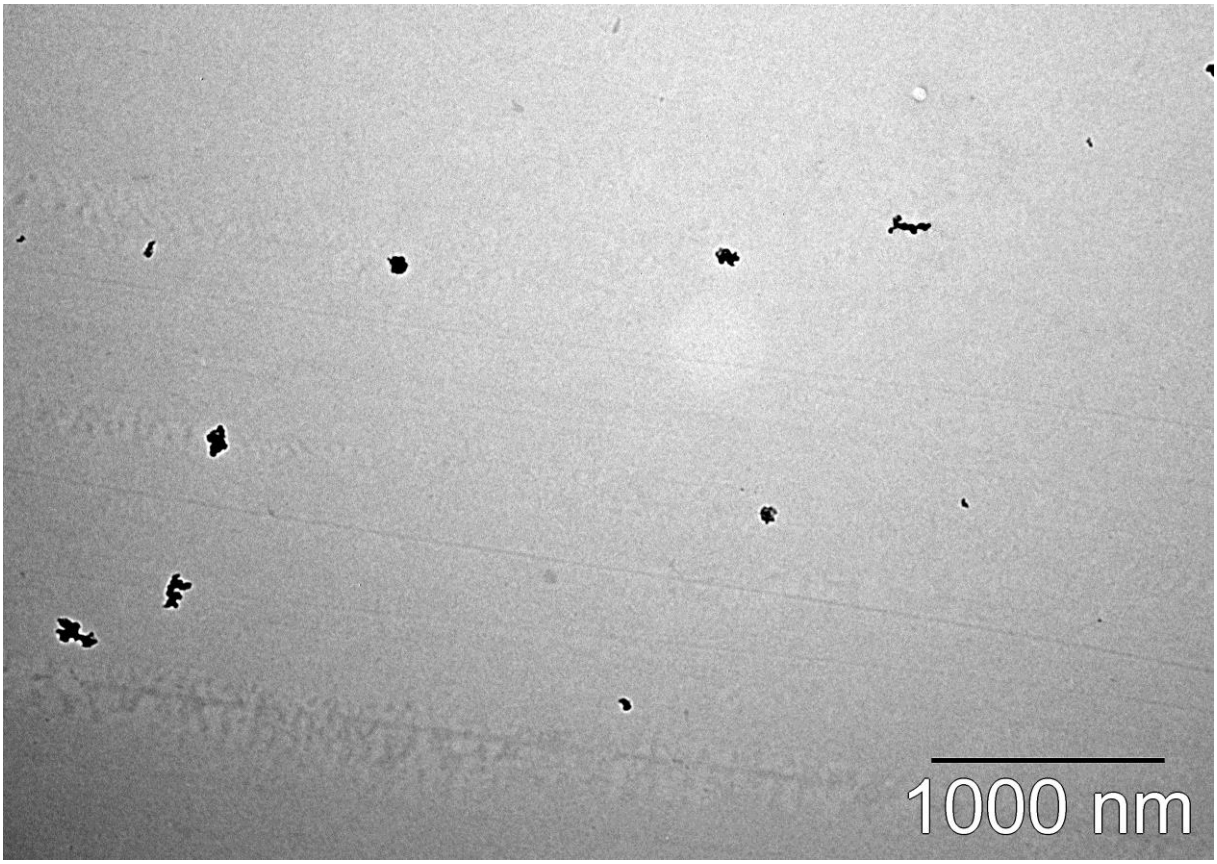
After post incubation, the cells (including the cell-culture inserts) were transferred into RNA protect buffer (Qiagen) and stored at 4°C until further use. RNA isolation was done with the RNeasy plus kit (Qiagen): the fixed cells were removed from the insert membrane by extensive vortexing. Complete displacement of the cells from the membrane was checked by eye. The cells were centrifuged at 9000 x *g* for 6 minutes and after removal of the RNA protect buffer, redissolved in 350 µl RLT plus buffer containing 1% β-mercaptoethanol. The cells were completely lysed by centrifuging them through Quashredder columns (Qiagen), followed by DNA removal by DNA elimination columns (Qiagen). The DNA-free lysates were further washed according to the RNeasy plus kit manual and the RNA was eluted in 30 µl RNA-free water, quantified (Naodrop) and immediately stored at -20°C. Reverse transcriptase reactions were performed in 10 µL volumes with an RNA concentration of 25 ng/µL (Omniscript RT, Qiagen) and Oligo dT primers (Qiagen). 0.25 µL RNase inhibitor (RNasin Plus RNase Inhibitor, Promega) was added to the reverse transcriptase reactions. A total of 2 µL of the ten-fold diluted cDNA was used for real-time PCR in reaction volumes of 10 µL with SYBR Green as reporter dye (Fast SYBR Green master mix, 7500 fast real-time PCR system, Applied Biosystems). Relative expression levels of heme-oxygenase (*HMOX1*), superoxide-dismutase (*SOD1*), tumor necrosis factor (*TNF*), interleukin-8 (*IL-8*), caspase7 (*CASP7*) and *FAS* were calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal standard gene. Primer sequences and database accession numbers are listed in Supporting Information Table S1.



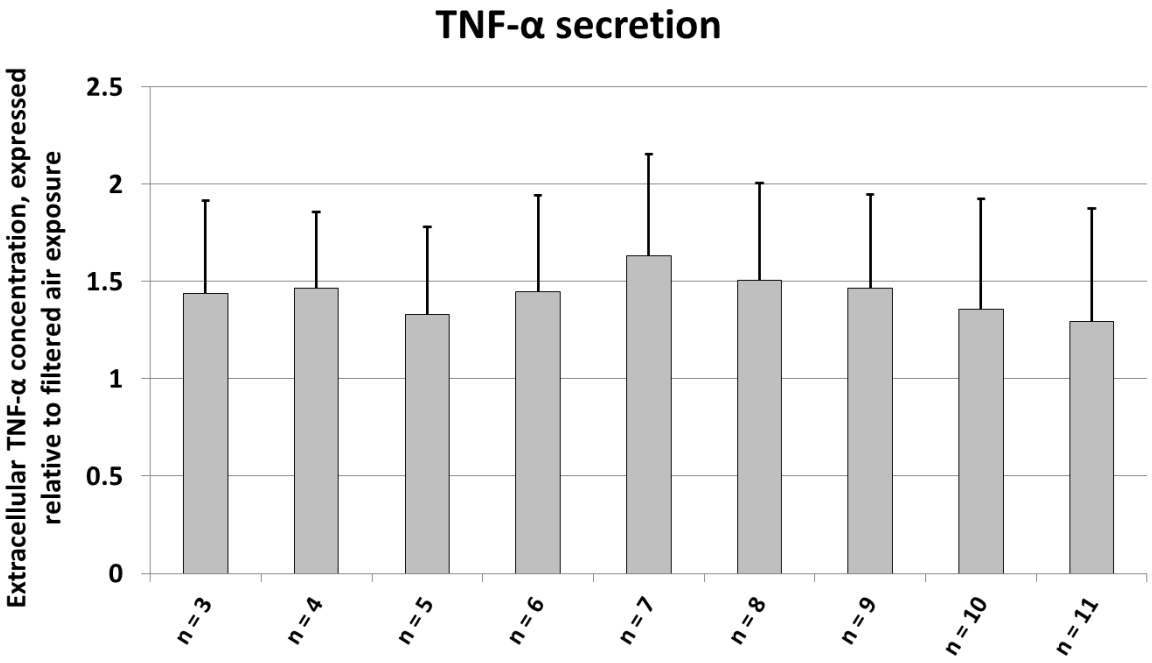
Supporting Information Figure S1: Schematic view of the used exposure system (adapted from Muller et al. 2010)



Supporting Information Figure S2: Size-number distribution of DEPs in filtered (red) and unfiltered (blue) ten-fold diluted diesel exhaust



Supporting Information Figure S3: A representative TEM picture of the diesel exhaust particles deposited in the exposure chamber. The TEM-grids were placed into the exposure chamber in a cell culture insert that was placed in an empty well of a cell culture plate during cell exposure experiments, hence at the exact same position as the cell cultures. The example shows deposited particles after 6 hours of exposure to unfiltered exhaust.



Supplemental Information Figure S4: Development of the standard deviation (SD) with increasing sample number. By increasing the sample number (exemplified here with the measurement of the extracellular TNF-α concentration upon 6 hours of exposure to unfiltered exhaust), the SD cannot be reduced, with the measured average being stable. We therefore conclude that the observed variation in the measured values is the variation intrinsic to the biological system, introduced by the presence of primary immune cells and indicative for the variation in the human population from which blood-donors were recruited.

Supporting Information Table S1: GenBank accession numbers of the analysed genes and sequences of the primers used for real-time PCR

Gene	GenBank accession	Direction	Primer sequence
GAPDH	NC_000012	forward	AAC AGC CTC AAG ATC ATC AGC
		reverse	GGA TGA TGT TCT GGA GAG CC
HMOX1	CP002685	forward	TTC TCC GAT GGG TCC TTA CAC T
		reverse	GGC ATA AAG CCC TAC AGC AAC T
SOD1	NM_000454	forward	GTG CAG GTC CTC ACT TTA AT
		reverse	CTT TGT CAG CAG TCA CAT TG
TNF	NM_000594	forward	CCC AGG GAC CTC TCT CTA ATC A
		reverse	GCT ACA GGC TTG TCA CTC GG
IL-8	NM_000584	forward	CTG GCC GTG GCT CTC TTG
		reverse	CCT TGG CAA AAC TGC ACC TT
CASP7	NM_001227	forward	CGG TCC TCG TTT GTA CCG TC
		reverse	GGT GGT CTT GAT GGA TCG CA
FAS	NM_000043	forward	AGC TTG GTC TAG AGT GAA AA
		reverse	GAG GCA GAA TCA TGA GAT AT

Supplemental Material Table S2: average values of all biological responses assessed over all experimental realizations. For quantification of extracellular LDH, cytokines and total reduced GSH/total protein, absolute values as well as the values normalized to the untreated control are shown. For GSH/protein, no unit can be assigned, LDH values are given in units of optical density at 490nm (n=10 for all raw exhaust data, 3 for gene expression data with filtered exhaust and 6 for the other filtered exhaust data)

			Values normalized to the untreated control				Absolute values			
			Unfiltered exhaust		Filtered exhaust		Unfiltered exhaust		Filtered exhaust	
			average	SD	average	SD	average	SD	average	SD
Extracellular LDH activity	2hrs	untreated control	1	± 0	1	± 0	0.9	± 0.3	1.1	± 0.4
		filtered air exposure	1.1	± 0.2	1.0	± 0.1	0.9	± 0.3	1.1	± 0.4
		exhaust exposure	1.0	± 0.1	1.2	± 0.2	0.9	± 0.4	1.2	± 0.4
	6hrs	untreated control	1	± 0	1	± 0	0.9	± 0.3	1.1	± 0.4
		filtered air exposure	1.2	± 0.4	1.1	± 0.2	1.0	± 0.3	1.3	± 0.5
		exhaust exposure	1.2	± 0.1	1.3	± 0.1	1.1	± 0.5	1.4	± 0.3
CASP7 expression	2hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	0.9	± 1.0	0.9	± 0.3				
		exhaust exposure	0.9	± 0.4	0.8	± 0.6				
	6hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	0.8	± 0.6	2.4	± 1.8				
		exhaust exposure	1.4	± 1.5	2.1	± 1.5				

Supplemental Material Table S2 continued

			Values normalized to the untreated control				Absolute values			
			Unfiltered exhaust		Filtered exhaust		Unfiltered exhaust		Filtered exhaust	
			average	SD	average	SD	average	SD	average	SD
FAS expression	2hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	1.1	± 0.9	0.9	± 0.8				
		exhaust exposure	1.3	± 0.9	1.3	± 1.5				
	6hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	0.6	± 0.3	1.3	± 0.7				
		exhaust exposure	0.5	± 0.2	1.3	± 0.5				
Total reduced GSH/total protein	2hrs	untreated control	1	± 0	1	± 0	0.0016	± 0.0011	0.0017	± 0.0004
		filtered air exposure	0.9	± 0.2	1.0	± 0.1	0.0015	± 0.0012	0.0017	± 0.0006
		exhaust exposure	0.1	± 0.1	0.2	± 0.1	0.0002	± 0.0003	0.0004	± 0.0002
	6hrs	untreated control	1	± 0	1	± 0	0.0015	± 0.0009	0.0016	± 0.0003
		filtered air exposure	0.9	± 0.2	1.0	± 0.3	0.0014	± 0.0010	0.0016	± 0.0007
		exhaust exposure	0.1	± 0.1	0.2	± 0.0	0.0002	± 0.0003	0.0003	± 0.0000
HMOX1 expression	2hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	1.1	± 0.6	1.4	± 0.3				
		exhaust exposure	24.11	± 15.83	32.64	± 22.43				
	6hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	3.3	± 4.1	1.2	± 0.4				
		exhaust exposure	84.08	± 70.68	21.22	± 17.66				

Supplemental Material Table S2 continued

			Values normalized to the untreated control				Absolute values			
			Unfiltered exhaust		Filtered exhaust		Unfiltered exhaust		Filtered exhaust	
			average	SD	average	SD	average	SD	average	SD
SOD1 expression	2hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	1.1	± 0.2	0.5	± 0.4				
		exhaust exposure	1.9	± 2.4	0.8	± 0.8				
	6hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	1.2	± 0.5	1.2	± 0.4				
		exhaust exposure	1.5	± 1.0	1.0	± 0.5				
TNF expression	2hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	0.9	± 0.4	2.5	± 2.5				
		exhaust exposure	1.5	± 1.7	2.1	± 1.3				
	6hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	1.0	± 0.6	1.7	± 1.0				
		exhaust exposure	1.7	± 1.0	1.7	± 1.4				
IL-8 expression	2hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	1.9	± 1.3	5.0	± 3.5				
		exhaust exposure	3.6	± 2.8	3.3	± 1.2				
	6hrs	untreated control	1	± 0	1	± 0				
		filtered air exposure	2.4	± 1.4	2.0	± 1.3				
		exhaust exposure	8.7	± 7.1	1.9	± 1.0				

Supplemental Material Table S2 continued

			Values normalized to the untreated control				Absolute values			
			Unfiltered exhaust		Filtered exhaust		Unfiltered exhaust		Filtered exhaust	
			average	SD	average	SD	average	SD	average	SD
TNF-α secretion	2hrs	untreated control	1	± 0	1	± 0	1.4	± 0.8 ng/ml	2.5	± 0.5 ng/ml
		filtered air exposure	2.8	± 2.0	2.2	± 0.5	3.2	± 2.3 ng/ml	5.3	± 1.2 ng/ml
		exhaust exposure	4.7	± 5.7	2.0	± 0.8	4.0	± 1.6 ng/ml	4.7	± 1.4 ng/ml
	6hrs	untreated control	1	± 0	1	± 0	1.6	± 0.5 ng/ml	2.1	± 0.7 ng/ml
		filtered air exposure	4.0	± 1.9	6.3	± 3.8	5.9	± 3.1 ng/ml	11.0	± 3.1 ng/ml
		exhaust exposure	5.1	± 2.6	5.0	± 3.4	7.2	± 3.0 ng/ml	8.6	± 2.3 ng/ml
IL-8 secretion	2hrs	untreated control	1	± 0	1	± 0	7.4	± 5.0 ng/ml	18.9	± 7.2 ng/ml
		filtered air exposure	1.4	± 0.5	1.1	± 0.1	9.4	± 4.9 ng/ml	20.0	± 5.7 ng/ml
		exhaust exposure	1.7	± 1.1	1.1	± 0.2	9.6	± 4.7 ng/ml	20.7	± 5.7 ng/ml
	6hrs	untreated control	1	± 0	1	± 0	9.7	± 2.8 ng/ml	18.8	± 7.2 ng/ml
		filtered air exposure	1.4	± 0.4	1.2	± 0.2	12.4	± 4.4 ng/ml	21.5	± 6.2 ng/ml
		exhaust exposure	1.6	± 0.8	1.1	± 0.2	14.1	± 5.5 ng/ml	19.7	± 7.2 ng/ml

Supporting Information Table S3: Comparison of the relative to filtered air exposure induced fold changes in the assessed biological responses (unfiltered exhaust vs. filtered exhaust). The values are obtained by dividing the fold change between filtered air and unfiltered exhaust exposure by the according value observed upon filtered exhaust exposure. The arrows indicate whether an effect is increased, decreased or unaffected by exhaust filtration. Asterisks indicate statistical significance ($p<0.05$)

	2 hours exposure					6 hour exposure				
	average		SD			average		SD		
Extracellular LDH activity	1.25	±	0.30	↑		1.17	±	0.20	↑	
CASP7 expression	0.92	±	0.62	→		0.71	±	0.65	↓	
FAS expression	1.03	±	0.36	→		1.30	±	0.70	↑	
levels of reduced GSH	2.67	±	0.86	↓	*	2.66	±	1.00	↓	*
HMOX1 expression	1.05	±	0.51	→		0.65	±	0.36	↓	
SOD1 expression	0.78	±	0.21	↓		0.63	±	0.09	↓	*
TNF expression	0.57	±	0.28	↓		0.60	±	0.21	↓	
IL-8 expression	0.40	±	0.11	↓	*	0.28	±	0.07	↓	*
TNF-α secretion	0.60	±	0.32	↓	*	0.64	±	0.18	↓	*
IL-8 secretion	0.87	±	0.08	→	*	0.77	±	0.13	↓	*

Supporting Information Table S4: Exhaust characterization of the ten-fold diluted diesel emissions and particle deposition in the cell exposure system

		Unfiltered exhaust			Filtered exhaust		
		Average		SD	Average		SD
Carbon monoxide (ppm)		33.01	±	1.91	31.38	±	1.37
Nitrogen oxides (ppm)		10.96	±	0.75	11.04	±	0.27
Nitrogen monoxide (ppm)		6.50	±	0.52	6.95	±	0.27
Nitrogen dioxide* (ppm)		4.38	±	0.30	4.09	±	0.09
Volatile hydrocarbons (ppm)		10.19	±	1.95	9.04	±	0.73
Particle number (particles/cm^3)		4.8×10^8	±	8×10^7	1.6×10^3	±	1.2×10^3
Elemental carbon ($\mu g/m^3$)		71.94	±	20.16	0.06	±	0.04
Total active surface area ($\mu m^2/cm^3$)		Out of range			1.12	±	0.37
Deposited particles ($/cm^2$)	2hrs	1.72×10^7	±	9.38×10^6	Below detection limit		
	6hrs	7.35×10^7	±	1.85×10^7	Below detection limit		

* NO_2 concentrations are based on: NO_x (ppm) - NO (ppm)

References

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